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REPORT #7

Host Defense Against Opportunistic Microorganisms Following Trauma

ANNUAL SUMMARY REPORT

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May 31, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick
Frederick, Maryland 21701

Contract No. DAMD 17-82-C-2165

The Christ Hospital Institute of
Medical Research
Cincinnati, Ohio 45219

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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. 78-23].



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SUMMARY

Circulating inhibitors of polymorphonuclear neutrophil, lymphocyte, and alternative complement pathway function that are produced following burn injury and are presumed to play an important role in lowering host resistance were studied in this investigation. Preliminary evidence suggesting that these inhibitory factors represent one molecular species was not confirmed. Infection was shown to be a major stimulus in induction of the inhibitors of neutrophil and complement function. This latter inhibitor was found to exert its effect by interfering with cleavage of C3 by alternative pathway convertases.

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I. OBJECTIVES

- A. To complete animal experiments designed to assess the relative roles of burn injury per se, wound colonization, and wound infection in induction of alterations of the complement system associated with burn injury.
- B. To develop methodology for the isolation of polymorphonuclear neutrophils from peripheral guinea pig blood and for measurement of the bactericidal activity of these leukocytes.
- C. To isolate the third component of complement, C3, from guinea pig plasma in chemically and functionally pure form for use in preparation of the C3d fragment and monospecific antiserum to this fragment.
- D. To confirm or refute preliminary data suggesting that burn-induced inhibition of alternative complement pathway activity, bacterial phagocytosis by polymorphonuclear neutrophils, and proliferative responses of lymphocytes to T cell mitogens is mediated by a common serum factor associated with alpha globulins.
- E. To identify the proteins of the alternative complement pathway that are affected by this putative inhibitor.
- F. To determine if certain arachidonate metabolites inhibit the functional activity of the alternative complement pathway and thus provide rationale for implicating these compounds in inhibition of complement function following burn injury.

II. BACKGROUND

Despite recent advances in the surgical and medical management of thermally injured patients, microbial infection continues to pose a severe threat to survival and, in fact, is the leading cause of death. Increased susceptibility to infection is related to loss of the protective skin barrier. In addition, burn injury induces profound abnormalities of the inflammatory and immune systems which are thought to further compromise host resistance. The intrinsic functions of "professional phagocytes" and T lymphocyte-dependent immune responses are adversely affected. Moreover, the molecules in circulation that modulate cellular functions undergo marked changes. These latter changes include reduction in the concentrations of immunoglobulins and fibronectin, alterations of the complement system, and appearance of circulating inhibitors that suppress vital functions of lymphocytes and phagocytes.

Our investigative efforts have been focused on the changes in the complement system induced by burn injury and their role in lowering host resistance against microbial infection. We have shown that the activity of C1-C9, as reflected by measurement of total hemolytic complement, and concentrations of individual components of the classical and alternative pathways often decline early after burn injury in patients with large full-thickness burns (1-4). This initial depression of the complement system resolves rapidly and, in fact, elevation of complement levels and C1-C9 activity is a trademark of the acute burn phase (1-4). Two of the complement proteins, however, do not return to normal or elevated concentration, i.e., properdin (P) and D¹ (1-8). In addition, an inhibitor

¹Nomenclature of the alternative complement pathway recommended by the World Health Organization (WHO Bulletin 59:489-491, 1981) has been used in this report.

becomes detectable in circulation that depresses the functional activity of the alternative complement pathway (2,5,6). It is not known whether this inhibitor exerts its effect through interaction with D, P, both proteins or by some other mechanism. Concentrations of the other alternative pathway proteins (C3, B, H, and I) are normal or elevated, and the ratio of the regulatory proteins to the proteins required for convertase formation, except D, is normal or reduced which favors activation of the alternative pathway rather than inhibition (8). Much to our surprise, we have shown that the complement alterations described above usually do not impede bacterial opsonization (1-4). But, occasionally, inhibitory activity has been detected in burn sera that depresses bacterial phagocytosis by polymorphonuclear neutrophils (7).

The cause-and-effect relationship between the appearance of the inhibitors described above and infection is presently unknown. In addition, the identity of these inhibitors and their modes of action are speculative. The report presented herein will focus on these unanswered questions.

III. PROGRESS REPORT

A. Animal Studies

During the preceding project period, we demonstrated that burn injury induces the same changes in the complement system of guinea pigs that occur in humans. These changes include a rapid rise in total hemolytic complement followed by a gradual reduction in the functional activity of the alternative complement pathway. Through use of the guinea pig model, we established that total hemolytic complement rises primarily in response to burn injury per se, whereas the abnormality of the alternative pathway develops in association with colonization and local infection of the burn wound with bacterial pathogens. We also found that invasive burn wound infection induced experimentally with bacteria or yeast exacerbates this latter abnormality, results in consumption of total hemolytic complement, and elicits production or release of factors into circulation that inhibit bacterial phagocytosis by polymorphonuclear neutrophils. During the present project period, we have completed determinations demonstrating that the inhibitory factors described above are not present in the sera of guinea pigs with 15% or 30% total body surface burns without systemic infection, indicating that invasive infection plays a major role in induction of these factors. In addition, we have shown that sera from the infected animals depressed phagocytosis through interaction with the leukocytes, a finding consistent with our previous observations in humans. Data from the animal experiments are presented in the preprint contained in the Appendix.

The polymorphonuclear neutrophils used in the studies described above were elicited by intraperitoneal injection of casein. These leukocytes have increased metabolic, phagocytic, and bactericidal activities as compared with non-elicited cells and therefore cannot be used in our future

experiments in which the effects of burn injury on the intrinsic functional activities of neutrophils will be examined. For this reason, we have evaluated five methods for preparation of non-elicited guinea pig neutrophils (9-13). These methods involve centrifugation of peripheral blood or leukocyte-rich plasma on continuous or discontinuous gradients of Hypaque-Ficoll or Percoll. Only one method, that by McCarthy et al. (13), was found to be successful. Final preparations contained approximately 70% neutrophils; yields were reasonable (1.0×10^7 cells per 25 ml of blood).

The bactericidal activity of these neutrophils has been measured using multiple bacterial strains and test conditions. The most informative results have been obtained using the method of Tan et al. (14), because, with the use of lysostaphin, intracellular killing as well as total killing by the neutrophils can be assessed. Reaction mixtures consisting of combinations of 1.25×10^7 Staphylococcus aureus 502A, 2.5×10^6 leukocytes, and 25% of pooled normal guinea pig serum in a final volume of 1 ml were incubated at 37°C. Aliquots were removed at 0 time and after 30, 60, and 120 minutes of incubation for measurement of total surviving bacteria. Surviving intracellular bacteria were also quantitated at the 120 minute time point. Phagocytosis and killing by the leukocytes occurred only when serum was present in the reaction mixtures (Table 1). Extracellular killing observed in non-rotated reaction mixtures was minimal.

Another goal of our future research is to determine the role of complement activation in depression of the immune system in our guinea pig model of burn injury. Complement activation will be assessed by quantitating circulating C3d by rocket immunolectrophoresis or radial immunodiffusion. During this project period, we have adapted the method of Tack et al. (15) to the isolation of C3 from guinea pig plasma. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (16) under reducing and non-

Table 1. Intracellular and Extracellular Bactericidal Activity of Peripheral Guinea Pig Polymorphonuclear Neutrophils for S. aureus 502A

Reactants	Total Killing (%)			Intracellular Bacteria (%)
	30 min.	60 min.	120 min.	120 min.
Serum, Leukocytes, Bacteria ^a	85.2	89.0	89.8	1.35
Leukocytes, Bacteria ^a	5.7	0	1.0	1.4
Serum, Leukocytes, Bacteria ^b	12.3	19.3	35.2	0.44

^aReaction mixtures were rotated at 37°C.

^bReaction mixtures were incubated at 37°C without rotation.

reducing conditions revealed bands which corresponded to the molecular weight of C3 (180,000 daltons) and its polypeptide chains (110,000 and 70,000 daltons) (Figure 1). The C3 preparation was also found to be homogeneous as assessed by Ouchterlony analysis using anti-whole guinea pig serum and anti-guinea pig C3. C3 hemolytic activity (17) on a weight basis was equal to that in guinea pig serum, indicating that C3 had been isolated with full retention of functional activity. We are now in the process of preparing the C3d fragment which will be used for preparation of monospecific antiserum. This antiserum will be used in development of immunochemical techniques for quantitation of C3d in guinea pig plasma.

B. Human Studies

Multiple immunosuppressive activities have been detected in human burn sera; however it is not known whether these activities are mediated by one molecular species or by many. Dr. John Mannick's research group has isolated a polypeptide of low molecular weight (<10,000 daltons) from the sera of burned patients and patients who have undergone major operative or accidental trauma that suppresses the proliferative response of normal human lymphocytes to T cell mitogens (18-20). This immunosuppressive factor occurs naturally in association with alpha globulins and, as a result, has been designated "immunoregulatory alpha globulin" (21). Its concentration is higher in the sera of injured patients than normal subjects (18-20). The factor also circulates in excess in patients with cancer (22). It has been postulated that diminished cellular immunity in the pathologic conditions described above is caused by increased production of immunoregulatory alpha globulin.

The role of this immunosuppressive factor in inhibition of complement and polymorphonuclear neutrophil function following burn injury is presently unknown. Alpha globulin can be fractionated from serum by DEAE-

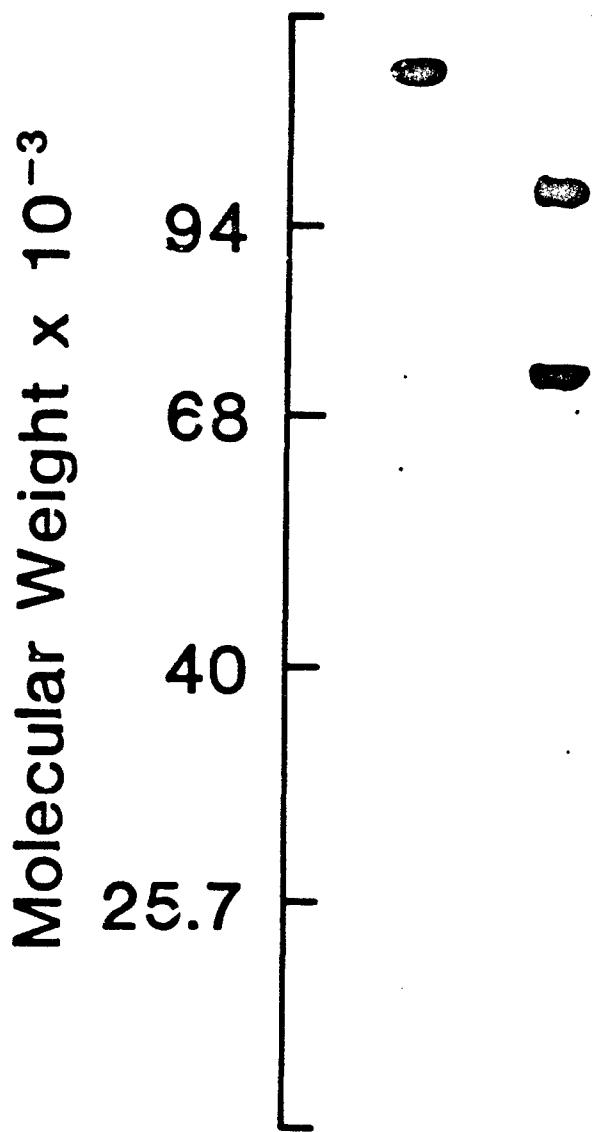


Figure 1. SDS polyacrylamide gel electrophoresis of isolated guinea pig C3. Non-reduced (left track) and reduced (right track) samples (4 μ g) were run on a 10% polyacrylamide slab gel. Marker proteins run simultaneously were phosphorylase a (mol. wt. 94,000), bovine albumin (mol. wt. 68,000), aldolase (mol. wt. 40,000), and α -chymotrypsinogen A (mol. wt. 25,700).

cellulose chromatography using the conditions described by Nimberg et al. (23). This chromatographic procedure also yields three other protein-rich fractions. In our Annual Summary Report (1982), the results of a pilot study were reported in which two human burn sera and pooled normal human serum were chromatographed as described above; the fractions were tested for their inhibitory effects on alternative complement pathway activity in human serum, bacterial phagocytosis by human polymorphonuclear neutrophils, and the proliferative response of human lymphocytes to concanavalin A. Maximal inhibition of complement, polymorphonuclear neutrophil, and lymphocyte function was observed with the alpha globulin fractions of the burn sera. The alpha globulin fraction of the normal serum inhibited lymphocyte function to a lesser extent and had no inhibitory effect on polymorphonuclear neutrophil or complement function. These results suggested that inhibitors of lymphocyte, neutrophil, and complement function in the burn sera might all be alpha globulin-associated factors.

During this project period, we have performed the experiments described above with DEAE-cellulose fractions prepared from three additional burn sera and three normal sera. Methodology described in detail in the preceding Annual Report was used (8); the fractions were tested at protein concentrations of 50 µg/ml. Alpha globulin fractions (peak 4) of the burn sera were not found to be more inhibitory on a weight basis than comparable fractions of the normal sera (Tables 2-4). Inhibition of complement, polymorphonuclear neutrophil, and lymphocyte function was not observed with unfractionated normal sera, yet alpha globulin fractions of two of these sera (control 1 and 2) inhibited all three functions. Moreover, unfractionated serum from patient 1 had marked inhibitory activity, yet this activity was not present in substantive amounts in fractions of this serum. These results taken together suggest that inhibitory material is not

Table 2. Inhibitory Effects of DEAE-Cellulose Fractions of
 Burn and Normal Sera on the Proliferative Response
 of Human Peripheral Lymphocytes to Concanavalin A

Serum Fractionated	Inhibition (%)			
	Peak 1	Peak 2	Peak 3	Peak 4
Patient 1	15.4	0	0	0
	2	17.2	0	0
	3	3.3	8.8	26.6
Control 1	17.8	9.6	0	94.2
	2	13.2	0	0
	3	22.9	6.3	1.1

**Table 3. Inhibitory Effects of DEAE-Cellulose Fractions of
Burn and Normal Sera on C3 Conversion in Pooled
Normal Human Serum**

Serum Fractionated	Inhibition (%)			
	Peak 1	Peak 2	Peak 3	Peak 4
Patient 1	10.5	7.7	0	6.3
	2	1.5	2.7	0
	3	44.3	29.9	15.8
Control 1	15.0	0	10.3	37.1
	2	N.D.*	N.D.	N.D.
	3	0	0	31.6

*N.D. = not determined.

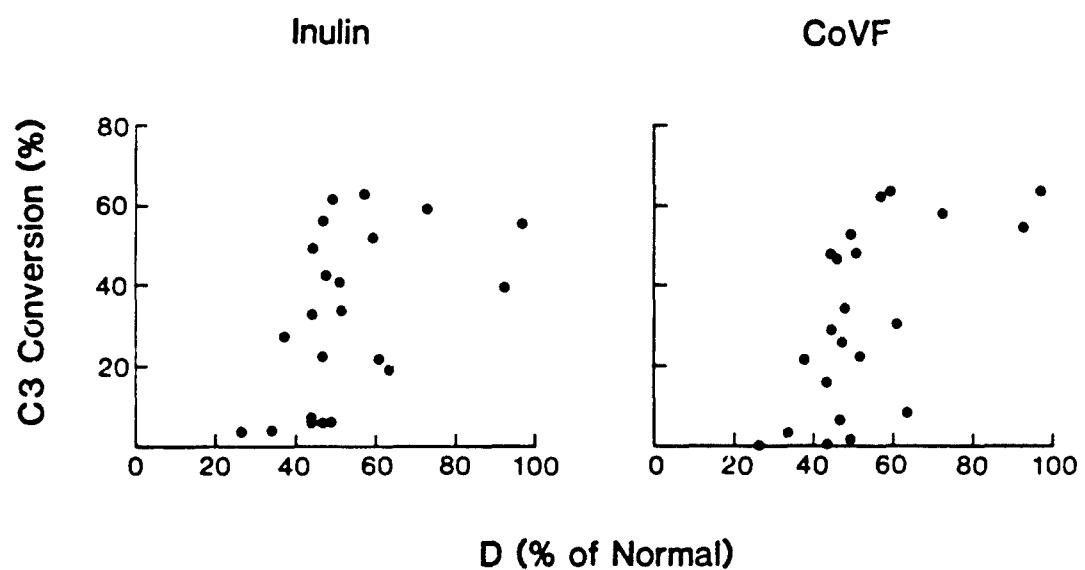
Table 4. Inhibitory Effects of DEAE-Cellulose Fractions of
Burn and Normal Sera on the Bactericidal Activity
of Human Polymorphonuclear Neutrophils

Serum Fractionated	Inhibition (%)			
	Peak 1	Peak 2	Peak 3	Peak 4
Patient 1	4.1	13.6	5.1	20.0
	2	7.2	0	9.0
	3	11.0	5.8	11.7
Control 1	0	0	50.5	39.5
	2	8.8	22.7	13.3
	3	N.D.*	N.D.	N.D.

*N.D. = not determined.

present in greater concentration in alpha globulin fractions of burn sera as compared with normal sera. The inhibitory material isolated in these fractions may represent endotoxin or other contamination resulting from the fractionation procedure. These results have prompted us to seek alternative approaches for the isolation and characterization of the burn serum inhibitors of interest.

Our investigation to identify the mechanism underlying inhibition of alternative complement pathway function associated with burn injury has included 11 patients (two sera each) and 12 healthy adult controls. Alternative pathway-mediated C3 conversion by inulin and cobra venom factor, immunochemical concentrations of C3, B, H, I, and P, and D activity have been measured in the sera. The methods used were identical to those described in the preceding Annual Report (8), except that the sera were treated with ethylene glycol bis-(β -aminoethyl ether)N,N'-tetraacetic acid to block classical pathway activity prior to measurement of C3 conversion. In accordance with our previous results, we found that reduction in alternative pathway-mediated C3 conversion is not related to elevation of the concentrations of H and I as compared with C3 and B. D was found to function abnormally in the burn sera, and the concentration of P was reduced. Yet, significant correlations were not demonstrated between D activity or P concentration in the burn sera and alternative pathway function (Figures 2 and 3). The relative concentrations of all six alternative pathway proteins, expressed as the ratio C3 + B + D + P/H + I, were also not found to correlate with alternative pathway function in the burn sera, even though these ratios were lower than those derived from control sera (Figure 4). These results indicated that the abnormal concentrations of the alternative pathway proteins in the burn sera were not directly responsible for reduction in alternative pathway activity.



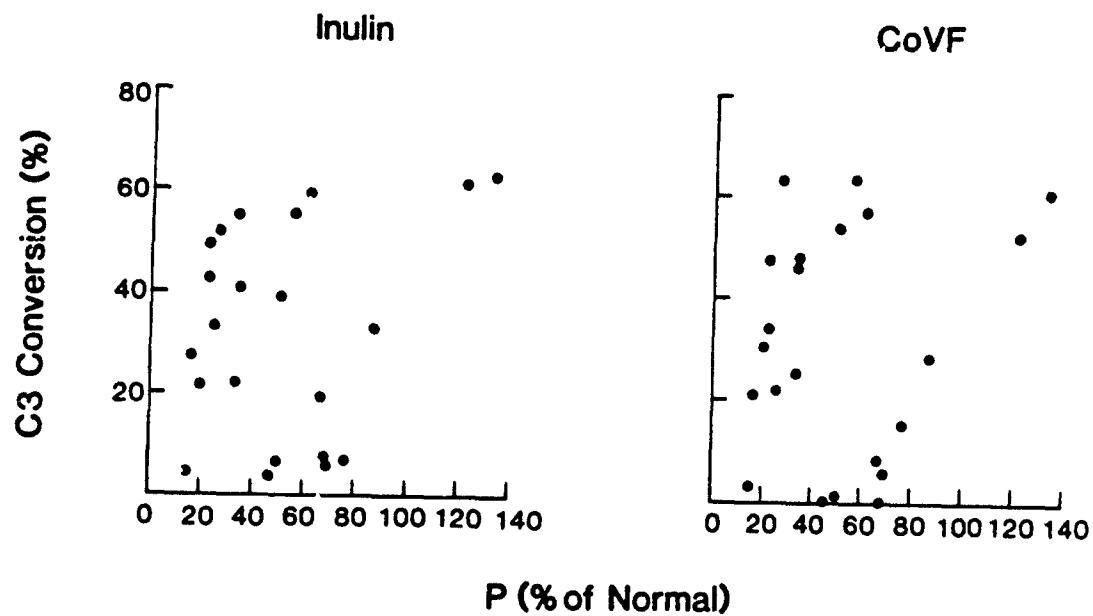


Figure 3. Relationship between C3 conversion by inulin and cobra venom factor and P concentration in the burn sera. Range of P concentration in the control sera was 66 to 122% (mean = 99%).

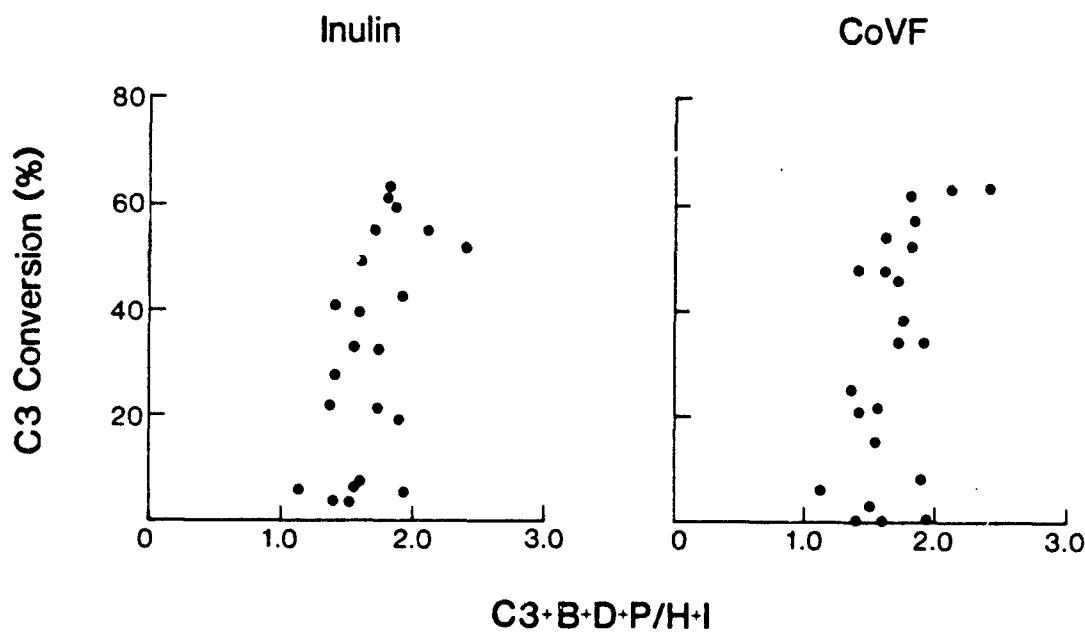


Figure 4. Relationship between C3 conversion by inulin and cobra venom factor and the ratio, C3 + B + D + P/H + I, in the burn sera. Ratios derived from the control sera ranged from 1.66 to 2.22 (mean = 2.04).

We have also measured concomitant C3 conversion by inulin and deposition of C3 fragments on this activator using burn and control sera supplemented with 11.5 μ g/ml of isolated radioiodinated normal human C3 (0.96% of the total C3). C3 was purified from normal human plasma by the method of Tack et al. (15). Radioiodination was carried out as described by Fraker and Speck (24) without loss of hemolytic activity; specific activity was 0.5 μ Ci/ μ g. C3 conversion was assessed by two methods, i.e., reduction in the B antigenic determinant of C3 and C3 hemolytic activity. By both methods, C3 conversion in the burn sera was markedly reduced as observed previously. However, radioiodinated C3 fragments were deposited equally well after incubation with burn or control sera. These results suggested that alternative pathway C3 convertases were formed in the burn sera that were able to cleave normal C3 but not burn C3. Perhaps, the substrate binding site of burn C3 for alternative pathway convertases is blocked by an inhibitor; as stated above, there is evidence in our previous work that an inhibitor is operative in alternative pathway dysfunction associated with burn injury.

Recent evidence suggests that burn injury induces increased synthesis of arachidonate metabolites that may play an important role in depression of the immune system (25,26). The influence of these compounds on the complement system is presently unknown. We theorized that prostaglandin (PG) E₁, PGE₂, or thromboxane B₂ might be involved in inhibition of the alternative complement pathway following burn injury. A series of pilot experiments was therefore performed to determine if these arachidonate metabolites interfered with alternative pathway function in normal human serum. Normal serum was supplemented with tenfold dilutions of commercially prepared PGE₁, PGE₂, and thromboxane B₂ ranging in concentration from 10⁻⁶ M to 10⁻⁹ M. Alternative pathway activity was measured by the

methods described in the preceding paragraph as well as by the method of Kaneko et al. (27); rabbit erythrocytes are employed as the alternative pathway activator in this latter assay. The arachidonate metabolites were not found to inhibit alternative pathway activity in any of the test systems, suggesting that they probably do not participate in alternative pathway dysfunction associated with burn injury.

IV. CONCLUSIONS

Burn injury and coexistent burn wound infection both play important roles in induction of humoral alterations of host defense associated with burn injury. Factors that depress bacterial phagocytosis by neutrophils appear in circulation only when burn wound infection is invasive. These factors are not alpha globulin-associated nor are the serum inhibitors of lymphoproliferative responses and alternative complement pathway activation. It is probable that multiple molecular species are involved in inhibition of neutrophil, lymphocyte, and complement function. The inhibitor of the alternative pathway appears to exert its effect by preventing cleavage of C3 by alternative pathway convertases and is distinct from PGE₁, PGE₂, and thromboxane B₂.

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VI. APPENDIX

Relative Roles of Burn Injury, Wound Colonization, and Wound Infection in
Induction of Alterations of Complement Function in a Guinea Pig Model of
Burn Injury

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Supported by the U.S. Army Medical Research and Development Command under contracts DAMD 17-81-C-1037 and DAMD 17-82-C-2165.

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Abstract

Stimuli involved in induction of alterations of the complement system and production of circulating inhibitor(s) of phagocytic function of polymorphonuclear neutrophils following burn injury were investigated using a guinea pig model of scald burn injury. The activity of C1-C9, assessed by measurement of total hemolytic complement, was found to increase primarily in response to burn injury per se, whereas reduction in the activity of the alternative complement pathway was shown to develop in association with natural colonization and local burn wound infection with bacterial pathogens. Invasive burn wound infection induced experimentally with Staphylococcus aureus, Pseudomonas aeruginosa, or Candida albicans exacerbated this latter abnormality, caused consumption of C1-C9 activity, and was associated with appearance of serum factors that depressed phagocytosis of Escherichia coli 075 by peritoneal polymorphonuclear neutrophils. Thus, injury and coexistent infection both play important roles in induction of humoral alterations of host defense associated with burn injury.

Introduction

Burn injury induces profound changes in the inflammatory and immune systems. Several comprehensive and detailed reviews have been written about these alterations and their potential biological significance (15, 16, 20). The intrinsic functions of "professional phagocytes" and T lymphocyte-dependent immune responses are adversely affected. In addition, the molecules in circulation that modulate cellular functions undergo marked changes. These latter changes include reduction in the concentrations of immunoglobulins and fibronectin, alterations of the complement system, and appearance of circulating factors that inhibit various functions of lymphocytes and phagocytes.

Our investigative efforts have been focused on the changes in the serum complement system induced by burn injury and their role in lowering host resistance against microbial infection. To date, our studies have been carried out exclusively in burned humans and, as a result, have been primarily descriptive in nature. We have shown that the activity of Cl-C9, as reflected by measurement of total hemolytic complement, and concentrations of individual components of the classical and alternative pathways decline early after burn injury in some patients with large full-thickness burns but not in others with comparable burn injury (2, 4-6). This initial depression of the complement system resolves rapidly and, in fact, elevation of complement levels and Cl-C9 activity is a trademark of the acute burn phase (2, 4-6). The only complement protein that does not return to normal or elevated concentration in circulation is properdin, and the properdin deficit is accompanied by reduction in the functional activity of the alternative pathway (2, 4-6). This latter abnormality worsens with bacteremia (7); bacteremic episodes have also been associated with consumption of Cl-C9 levels resulting in a decrease in hemolytic activity from the supranormal to

normal or subnormal range (2, 4-6). Much to our surprise, we have shown that the complement alterations described above do not usually impede bacterial opsonization (2, 4-6). But, occasionally, inhibitory activity has been detected in burn sera that depresses bacterial phagocytosis by polymorphonuclear neutrophils (8).

The stimuli involved in production of this neutrophil inhibitor and induction of the changes in the complement system described above are the subject of the present investigation. A guinea pig model of scald burn injury has been used to assess the relative roles of injury, wound colonization, and wound infection in induction of these humoral alterations. The guinea pig was selected for our study, because the complement system of this animal closely resembles that of the human (9, 10, 13, 21, 23). In addition, controlled studies have shown that the metabolic response of growing guinea pigs to severe burn injury simulates the human postburn metabolic response (14).

MATERIALS AND METHODS

Animals. Male and female Hartley guinea pigs (300-350 g) were purchased from Murphy Breeding Labs, Inc., Plainfield, IN. The animals were housed in separate cages and allowed to adapt to the new environment for 10 days. The animals were fed Ralston Purina guinea pig chow ad libitum before and after burning or sham-burning. In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. 78-23].

Burning and Sham-Burning of Animals and Induction of Experimental Infection. A modification of the method of Herndon et al. (14) for burning and

sham-burning guinea pigs was used. Three hundred and fifteen animals were divided into three groups. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight) (Veterinary Laboratories, Inc., Lenexa, KA). The animals were weighed, and their hair was clipped. Scald burns were applied to two groups by placing the animals in a custom-made insulated mold, which exposed a 60 cm² area on the dorsum equal to approximately 15% of the total body surface (TBS). The area was immersed in 99°C water for 13 seconds. Due to the increased rate of heat loss associated with thermal injury, the burned animals were placed on heating blankets to reduce the stress associated with this phenomenon. After 1 hour, the animals in one of the two groups were again placed in the mold, and a second dorsal burn was applied. The animals were removed from the mold and replaced on the heating blankets. Animals with 30% TBS burns received 50 ml/kg of lactated Ringer's solution (Abbott Laboratories, North Chicago, IL) immediately before injury and at 1.5 and 5 hours after injury. Animals with 15% TBS burns received 25 ml/kg of lactated Ringer's solution immediately before injury and at 3 hours after injury. The third group of animals served as the sham-burned controls. This group was handled identically to the group receiving 15% burns, with the exception that the animals were immersed in tepid water and received the second dose of lactated Ringer's solution on the morning following the procedure. Survival on the day following burning or sham-burning was 98%. Autopsies were performed on selected burned animals 24 hours following injury. The results demonstrated that full-thickness injuries had been produced without damage to the visceral organs.

Experimental infection was induced in three groups of guinea pigs with 30% TBS burns (12-14 animals per group). Immediately following burning, the animals in each group were injected subcutaneously under the burn wound with

5.0×10^5 colony forming units (cfu) of Staphylococcus aureus, Pseudomonas aeruginosa, or Candida albicans. The strains used were blood culture isolates from burned humans and were grown for 16 hours at 37°C in trypticase soy broth (BBL, Cockeysville, MD) (S. aureus and P. aeruginosa) or Sabouraud dextrose broth (Difco Laboratories, Detroit, MI) (C. albicans). The organisms were washed once with Hank's balanced salt solution (M.A. Bioproducts, Walkersville, MD) containing 0.1% gelatin (Difco Laboratories) (GHBSS) before use.

Procurement and Processing of Cultures and Sera. The groups of animals sacrificed from 5 to 50 days postburn were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight), weighed, and bled aseptically by cardiac puncture. The groups sacrificed on the day of burning or sham-burning were not reanesthetized or reweighed, since blood was drawn within 2 hours following the procedure. Blood was allowed to clot in glass tubes for 1 hour at room temperature and up to 4 hours at 4°C. The tubes were centrifuged at 5000 x g for 10 minutes at 4°C, and the sera were removed. The sera were divided into small aliquots and frozen at -70°C.

Quantitative burn wound cultures were obtained using the method of Saymen et al. (24). Burn eschar was incised, and a small specimen of subjacent tissue including panniculus muscle at the center of the burn wound was removed by sharp dissection. The specimens were transferred aseptically to pre-weighed sterile capped polystyrene tubes (12 x 75 mm; Falcon Plastics, Oxnard, CA) and weighed. One ml of sterile physiologic saline, pH 7.0, was added to each tube in order to transfer the specimen to a 30 ml Potter Elvehjem tissue grinder. The tube was rinsed with 1 ml of sterile saline into the tissue grinder. The loaded grinder was immersed in an ice bath, and the tissue was homogenized for 10 to 15 minutes using a drill press. The total number of bacteria in each specimen was determined by a standard

dilution plate count method using sheep blood agar as the growth medium. After 24 hours of incubation at 35°C, individual colony types were counted and Gram-stained. The bacteria were identified by standard methods (18). The number of colony forming units (cfu) of individual bacterial species in each specimen was estimated by multiplying the proportion of that species as assessed by colonial morphology by the total number of cfu. Results were expressed as log₁₀ cfu per 100 mg of specimen.

After procurement of specimens, animals were euthanized by intraperitoneal injection of T-61 euthanasia solution (1 ml/kg body weight) (American Hoechst Corp., Somerville, NJ).

Complement Determinations. Total hemolytic complement was titrated by the method of Mayer (19) using a volumetric modification. For measurement of alternative pathway activity, sera were incubated for 5 minutes at 37°C with 10 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA; Sigma Chemical Co, St. Louis, MO) to block the classical pathway and leave the alternative pathway intact (11). Fifty-five and one half μl of inulin (100 mg/ml) (Sigma Chemical Co.) were added to 500 μl of EGTA-treated serum in 12 x 75 mm polystyrene tubes (Falcon Plastics). Physiologic saline, pH 7.0, was substituted for inulin in the controls. One hundred and eleven μl samples were removed at 0 time and after 10 minutes of incubation at 37°C. The samples were centrifuged at 1600 x g for 5 minutes at 4°C, and residual total hemolytic complement was titrated in the supernatants as described above. The results were expressed in percent of complement consumption and were calculated by the formula $a-b/a \times 100$, where a was equal to total hemolytic complement at 0 time and b was equal to total hemolytic complement at 10 minutes.

Measurement of Serum Phagocytosis-Promoting Activity. The method of Leist-Welsh and Bjornson (17) was adapted for measurement of phagocytosis-

promoting activity in guinea pig sera. Sera were tested at concentrations of 10% and 98%. Stock cultures of Escherichia coli 075 were maintained in trypticase soy broth at -70°C. Five μ l of thawed culture were inoculated into 1 ml of trypticase soy broth containing 10 μ Ci/ml of [methyl-³H] thymidine (5 Ci/mmol; Amersham, Arlington Heights, IL) and incubated for 16 hours at 37°C. Bacteria were pelleted at 5000 \times g for 10 minutes at 4°C, washed once, and suspended in GHBSS to a final concentration of 2.5×10^9 cfu/ml. Incorporation ranged from $2.3-3.6 \times 10^4$ counts per minute (cpm) per 5.0×10^7 cfu. Peritoneal exudates were induced in non-burned 300-350 g Hartley guinea pigs by injection of 15-20 ml of 12% casein (Fisher Scientific Co., Fair Lawn, NY) in sterile physiologic saline, pH 7.4, and collected 18-20 hours later by lavage with 50-70 ml of GHBSS containing 10 units/ml of heparin (Elkin-Sinn, Inc., Cherry Hill, NY). The leukocytes were washed twice and suspended in GHBSS to a final concentration of 5.0×10^7 cells/ml. The leukocyte suspensions contained 89% to 92% polymorphonuclear neutrophils and 8% to 11% mononuclear cells. Five hundred μ l of leukocyte suspension containing 2.5×10^7 cells were added to 12 \times 75 mm capped polyethylene tubes (Falcon Plastics), and the tubes were centrifuged at 225 \times g for 7 minutes at 4°C. The supernatants were discarded, and 50 μ l or 490 μ l of test serum, 10 μ l of bacterial suspension (2.5×10^7 cfu), and GHBSS were added to the leukocyte pellets in a final volume of 500 μ l. Controls consisting of serum and bacteria were prepared in tubes lacking leukocytes, and controls consisting of leukocytes and bacteria were prepared by substitution of GHBSS for the serum. The tubes were gently vortexed and then tumbled at 37°C on a rotating platform. After 10 minutes of incubation, aliquots (100 μ l) were removed to 3 ml of GHBSS containing 0.01 M sodium fluoride (Sigma Chemical Co.). Leukocyte-associated bacteria were separated from non-associated bacteria by centrifugation at 120 \times g for 5 minutes at

4°C. The leukocyte pellets were washed three times with 3 ml of GHBSS containing sodium fluoride. The final leukocyte pellets were solubilized in 500 μ l of NCS tissue solubilizer (Amersham) for 40 minutes at 50°C. Glacial acetic acid (17 μ l) was added, and the samples were transferred to glass mini-scintillation vials (Kimble Corp., Toledo, OH). A 5 ml volume of organic counting scintillant (Amersham) was added, and the samples were counted in a Beckman LS 7000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Samples (100 μ l) were also removed after 10 minutes of incubation from controls lacking leukocytes. The samples were centrifuged at 12,000 \times g for 20 minutes at 4°C to deposit the bacteria for measurement of total cpm. The pellets were solubilized and counted as described above. The percent of uptake was calculated by dividing the cpm in the leukocyte pellets by the total cpm and then multiplying by 100. Preliminary experiments indicated that uptake of E. coli 075 by the leukocytes in the presence of serum was accompanied by intracellular killing; uptake and killing were not demonstrated in the absence of serum.

In some experiments, bacteria were preopsonized with 98% of the test sera by incubating 7 μ l of bacteria (1.75×10^7 cfu) for 15 minutes at 37°C with 343 μ l of serum. GHBSS was substituted for the serum in the controls. The bacteria were then pelleted by centrifugation at 12,000 \times g for 20 minutes at 4°C, washed once with 2 ml of GHBSS, and resuspended in 175 μ l of GHBSS. One hundred and seventy-five μ l of leukocytes containing 1.75×10^7 cells were added, and a 100 μ l aliquot was removed immediately for measurement of total cpm. Leukocyte-associated radioactivity was measured after rotation for 10 minutes at 37°C. The percent of uptake was calculated as described above.

Statistical Analyses. Differences in the data were analyzed by analysis of variance (31). Duncan's multiple range test was used to further describe

the differences (28). Results from the assay of complement consumption and phagocytosis-promoting activity and the quantitative culture results were transformed before computing analysis of variance. The transformations used were as follows: (a) arcsin (complement consumption), (b) \log_e (phagocytosis-promoting activity), and (c) square root (quantitative cultures) (29). A probability value of less than 0.05 was considered to be significant.

RESULTS

Survival, Change in Weight, and the Temporal Sequence of Burn Wound Colonization in Non-Experimentally Infected Animals. Survival of animals during 50 days postburn was as follows: (a) 84%, 30% TBS burns; (b) 96%, 15% TBS burns, and (c) 98%, sham-burned. Mortality of animals with 30% burns occurred between 15 and 50 days postburn, and mortality of animals with 15% burns occurred between 20 and 40 days postburn.

Marked weight loss was observed during 10 days postburn in the animals with 30% burns (Fig. 1). The weights of the animals with 15% burns remained relatively constant during 5 days postburn and increased thereafter. Weight gain was observed in the control animals at 5 days following sham-burning and on all subsequent days.

The time course of colonization of the burn wounds in the two groups of burned animals was similar (Fig. 2). Immediately following injury, few bacteria were isolated from burn wound tissue. By 10 days postburn, all animals in both burned groups were heavily colonized. Colonization persisted in all animals during 50 days postburn. Gross infection characterized by malodorous, purulent exudate and necrosis of subcutaneous tissue was evident from 10 through 35 days postburn. The most common infecting agent was S. aureus; other isolates were Staphylococcus epidermidis, alpha streptococci, Acinetobacter calcoaceticus, Proteus vulgaris, E. coli, P.

aeruginosa, Aeromonas hydrophila, Citrobacter species, Shigella species, and Bacillus species.

The frequency of isolation of Gram-positive cocci from the two groups of burned animals was similar except on day 5, when a greater frequency of isolation was noted in the animals with 15% burns (Table I). The numbers of these bacteria isolated from the two groups of animals after day 5 were not significantly different. Similar results were obtained when the data were analyzed according to the frequency of isolation and the number of S. aureus isolated from the two groups of animals, with the exception that after day 5 S. aureus was isolated with greater frequency from the animals with 30% burns.

The frequency of isolation of Gram-negative rods from the two burned groups was quite different (Table I). On day 10, these bacteria were isolated with greater frequency from the animals with 15% burns, but by day 20 only one of eight of these animals had positive cultures as compared with five of six animals with 30% burns. After day 20, colonization with Gram-negative rods persisted in 28% of the animals with 30% burns but was not observed in the animals with 15% burns.

Complement and Phagocytosis-Promoting Activity in Sera From Non-Experimentally Infected Animals. Total hemolytic complement was significantly increased in both groups of burned animals as compared with the sham-burned controls beginning at 5 days postburn (Fig. 3). Total hemolytic complement peaked on day 20 and returned to normal more rapidly in the animals with smaller burns; significant differences in the data from the two burned groups were observed on days 25 and 30. This latter observation and the lack of temporal association with burn wound colonization (Fig. 2) suggest that total hemolytic complement rises primarily in response to burn injury per se.

Alternative complement pathway activity was significantly decreased in the animals with 30% burns as compared with the control animals and the animals with 15% burns (Fig. 4). The onset of this abnormality occurred at 10 days postburn and coincided with heavy colonization of the burn wounds with bacteria (Fig. 2). Alternative pathway function began to normalize on day 40 as gross local infection subsided.

Sera from the animals with 30% burns and the controls facilitated comparable uptake of *E. coli* 075 by normal guinea pig peritoneal polymorphonuclear neutrophils at all days postburn when tested at concentrations of 10% or 98% (Table II). Significant differences were also not observed when the data from the animals with 15% burns and the controls were compared (results not shown).

Survival, Weight Loss, and Serum Complement and Phagocytosis-Promoting Activity Following Experimental Burn Wound Infection. Survival at 5 days post-infection was 50% (*S. aureus*), 33% (*P. aeruginosa*), and 57% (*C. albicans*). No deaths occurred during 5 to 13 days postburn. Mean weight loss during this period was as follows: -54 g (*S. aureus*), -66 g (*P. aeruginosa*), and -60 g (*C. albicans*). Total hemolytic complement in the sera of the majority of survivors fell within or below the range of control values during the 5 to 13 day period of study (Fig. 5). Alternative pathway activity in the sera of all experimentally infected animals was markedly reduced as compared with the control values (Fig. 6). Testing of phagocytosis-promoting activity for *E. coli* 075 using a serum concentration of 10% revealed that the majority of the animals had normal phagocytosis-promoting activity as compared with the controls (Fig. 7). However, reduction in phagocytosis-promoting activity was demonstrated when the sera from the experimentally infected groups were tested at a concentration of 98% (Fig. 8). Comparison of the values derived from experimentally infected and

naturally colonized animals with 30% burns during 5-15 days postburn showed that total hemolytic complement, alternative pathway-mediated complement consumption, and phagocytosis-promoting activity (98% serum concentration only) were lower in the experimentally infected groups relative to their respective control values.

The six sera obtained on day 7 from the experimentally infected animals were tested at a concentration of 98% for their ability to opsonize *E. coli* 075. Mean uptake \pm SEM by peritoneal polymorphonuclear neutrophils of bacteria opsonized with sera from infected animals was $53 \pm 5\%$ as compared with $61 \pm 10\%$ using bacteria opsonized with pooled normal guinea pig serum; these data were not significantly different. These results suggest that sera from the infected animals depressed phagocytosis through interaction with the leukocytes.

DISCUSSION

The results of our investigation show that burn injury induces the same changes in the complement system of guinea pigs that occur in humans. These changes include a rapid rise in total hemolytic complement followed by a gradual reduction in the functional activity of the alternative complement pathway. Through use of the guinea pig model, we have established that total hemolytic complement rises primarily in response to burn injury per se, whereas the abnormality of the alternative pathway develops in association with colonization and local infection of the burn wound with bacterial pathogens. We have also provided evidence that invasive burn wound infection with bacteria or yeast exacerbates this latter abnormality, results in consumption of total hemolytic complement, and elicits production or release of factors into circulation that inhibit bacterial phagocytosis by polymorphonuclear neutrophils. The mechanisms by which burn injury and coexistent

burn wound infection induce the humoral alterations described above remain to be elucidated.

Our data suggest that acquisition of the abnormality of the alternative pathway is influenced by the size of the burn wound and the type and number of colonizing bacteria. Guinea pigs with 30% TBS burns developed this abnormality only after their burn wounds had become heavily colonized with bacteria. As colonization with S. aureus and Gram-negative rods persisted, the abnormality worsened. Guinea pigs with smaller burns colonized with proportionally fewer bacteria of generally less pathogenic species did not acquire the abnormality. It should be noted that a direct relationship was not demonstrated between the number or type of bacteria colonizing the animals with 30% burns and the extent of reduction in alternative pathway function. The best indicator of the presence of this abnormality was the appearance of gross local infection.

In accordance with our previous results in humans (2, 4-6), the abnormality of the alternative pathway in the burned guinea pigs was not found to compromise serum opsonic activity. The bacterial test strain used in our investigation for measurement of opsonic activity was E. coli 075, which has been shown to activate the alternative pathway resulting in deposition of opsonic C3 fragments onto the bacterial surface (3, 25). It is not known why opsonization of this bacterium was able to proceed normally despite reduction in alternative pathway function. It is doubtful that a fibronectin-mediated mechanism of opsonization compensated for the complement abnormality, since gelatin was present in our assay system and would have saturated available fibronectin in the serum. Previously, we speculated that opsonic antibodies produced during burn wound colonization might augment the poorly functioning alternative pathway, but this hypothesis has been subsequently ruled out (6). The classical pathway may be activated

preferentially during opsonization circumventing the alternative pathway abnormality. Alternatively, the abnormality may be a fluid phase phenomenon that is inoperative in surface-mediated assembly of alternative pathway convertases. We are actively investigating these latter possible mechanisms.

Appearance of factors in circulation that depress bacterial phagocytosis by neutrophils was shown in our investigation to be associated with consumption of total hemolytic complement from the supranormal to normal range occurring during invasive burn wound infection. It is well documented that complement cleavage products cause aggregation of polymorphonuclear leukocytes, excessive exocytosis of lysosomal enzymes, and secondary unresponsiveness to chemotactic and endocytic stimuli (12, 22, 26, 27, 30). The possibility that "burn serum inhibitors" of neutrophil function are complement cleavage products warrants further investigation.

Our previous results have suggested that alternative pathway dysfunction associated with burn injury is also related to an inhibitory factor (1, 2, 7). The evidence for this theory was derived from experiments in which mixture of equal parts of burn serum and normal serum was not found to correct the abnormality. The levels of the alternative pathways proteins in human burn sera, with the exception of properdin, have been shown to be normal or supranormal, which suggests that alternative pathway dysfunction is not related to consumption of the proteins essential for convertase formation (2, 4-6). Although complement consumption does occur during systemic infection as was observed in our experimentally infected animals, it appears to be compensated for by increased synthesis of complement proteins. The rate of synthesis of C3 and B keeps up with that of H and I (unpublished observation). Thus, reduction in alternative pathway function is not related to an imbalance of the ratio of control proteins to the

proteins required for convertase formation. Increased synthesis of complement proteins may be accompanied by production of an as yet undefined regulatory factor that facilitates the action of H and I or dampens alternative pathway activation by another mechanism. The identity of the inhibitory factor and its mode of action are currently under investigation in our laboratory.

The guinea pig model of burn injury used in this investigation has been previously shown to simulate the burned human with respect to metabolic alterations [14]. Our results demonstrate that the temporal sequence of burn wound colonization in this model is also similar to that occurring in man. The low mortality observed in naturally colonized, burned guinea pigs is probably related to their age and the virulence of the colonizing bacteria. The high mortality associated with experimental infection of the burn wound with human blood culture isolates may be related to the greater virulence of these microorganisms. Pathogens present in the hospital environment would be expected to be highly virulent, since they have been transferred from one human to another and thus have been subjected to a form of "animal passage." Alternatively, inoculation of the microorganisms directly into the tissues rather than onto the surface of the burn wound, as occurs naturally, may have circumvented local host defense mechanisms resulting in fulminant infection. The fortuitous finding that the guinea pig model has an excellent survival rate during 50 days postburn without therapeutic intervention warrants its use in additional investigations of immunologic abnormalities associated with burn injury.

Acknowledgments

The technical assistance of Lori Jones, Annette Collier, and Sue Lewis, the statistical consultations by Kathy Dietrich, and the secretarial assistance of Marcia Bacon are gratefully acknowledged.

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TABLE I

Frequency of isolation and number of Gram-positive cocci, *S. aureus*, and Gram-negative rods per 100 mg of burn wound tissue in animals with 15% (Group 1) and 30% (Group 2) TBS burns

Days Postburn	Animals with Positive Cultures/Total Animals (Range of cfu per 100 mg Tissue)*					
	Gram-Positive Cocci		<i>S. aureus</i>		Gram-Negative Rods	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
0	1/9 (1.3)	1/10 (1.6)	0/9		1/10 (1.6)	0/9
5	7/8 (1.78-7.68)	4/10 (3.6-4.6)	6/8 (1.6-7.68)		1/10 (3.78)	2/8 (1.3-3.13)
10	6/8 (3.3-7.55)	7/9 (5.33-7.90)	1/8 (5.6)		7/9 (7.0-7.9)	7/8 (5.6-7.31)
15	6/9 (4.58-7.64)	9/9 (5.3-7.6)	2/9 (3.6-6.81)		8/9 (5.3-7.6)	5/9 (5.3-6.76)
20	6/8 (5.6-7.81)	3/6 (7.6-8.73)	4/8 (5.3-7.6)		3/6 (7.3-8.73)	1/8 (7.51)
25	6/7 (5.66-7.63)	4/5 (6.0-8.41)	5/7 (4.72-7.29)		3/5 (6.85-8.41)	0/7 (6.34-8.51)
30	7/7 (4.73-7.62)	6/6 (5.3-7.96)	7/7 (5.6-7.62)		5/6 (6.41-7.96)	0/7 (3.3-6.3)
35	6/7 (5.9-7.5)	7/7 (6.0-7.6)	2/7 (5.3-5.6)		6/7 (5.78-7.6)	0/7 (5.9)
40	9/9 (5.3-8.14)	6/6 (6.15-7.6)	6/9 (5.38-8.14)		6/6 (6.15-7.6)	0/9 (5.6)
45	10/10 (6.15-7.56)	8/8 (5.66-9.0)	10/10 (1.22-7.56)		8/8 (5.66-9.0)	0/10 (5.78-6.15)
50	8/8 (6.34-7.48)	7/7 (6.26-7.53)	3/8 (6.34-7.09)		7/7 (6.26-7.53)	0/8 (5.78-6.15)

*Results are expressed as log₁₀.

TABLE II

Uptake of *E. coli* 075 by normal guinea pig peritoneal polymorphonuclear neutrophils
in the presence of sera from animals with 30% TBS burns and sham-burned animals

Days Postburn	Percent Uptake* (No. Animals)			
	10%†		98%†	
	Sham-Burned	30% TBS Burns	Sham-Burned	30% TBS Burns
0	78.3 ± 4.3 (8)	74.8 ± 9.9 (5)	72.2 ± 4.3 (8)	69.1 ± 7.5 (6)
5	73.4 ± 5.3 (10)	94.8 ± 8.3 (4)	77.5 ± 4.1 (9)	75.6 ± 7.5 (6)
10	82.3 ± 3.1 (9)	87.7 ± 4.6 (8)	71.4 ± 3.2 (8)	67.6 ± 3.7 (8)
15	77.8 ± 5.0 (8)	72.9 ± 5.3 (7)	64.7 ± 4.1 (7)	57.4 ± 3.0 (6)
20	75.1 ± 4.6 (10)	96.2 ± 14.1 (6)	82.0 ± 4.9 (8)	71.6 ± 7.4 (6)
25	81.4 ± 1.5 (9)	39.1 ± 8.4 (7)	78.6 ± 3.4 (7)	69.2 ± 5.2 (7)
30	85.5 ± 9.0 (9)	88.4 ± 6.6 (8)	76.5 ± 4.9 (8)	74.8 ± 3.5 (7)
35	80.1 ± 3.4 (10)	79.3 ± 9.7 (7)	77.9 ± 5.3 (10)	75.4 ± 8.1 (6)
40	81.2 ± 5.1 (9)	87.3 ± 5.1 (6)	74.7 ± 3.3 (9)	73.6 ± 4.9 (5)
45	85.7 ± 8.4 (10)	79.3 ± 7.3 (6)	69.1 ± 4.9 (9)	72.3 ± 5.9 (6)
50	76.0 ± 4.6 (7)	80.0 ± 6.8 (7)	75.5 ± 6.9 (7)	70.8 ± 4.4 (6)

*Mean values ± SEM.

†Concentration of serum used in the assays.

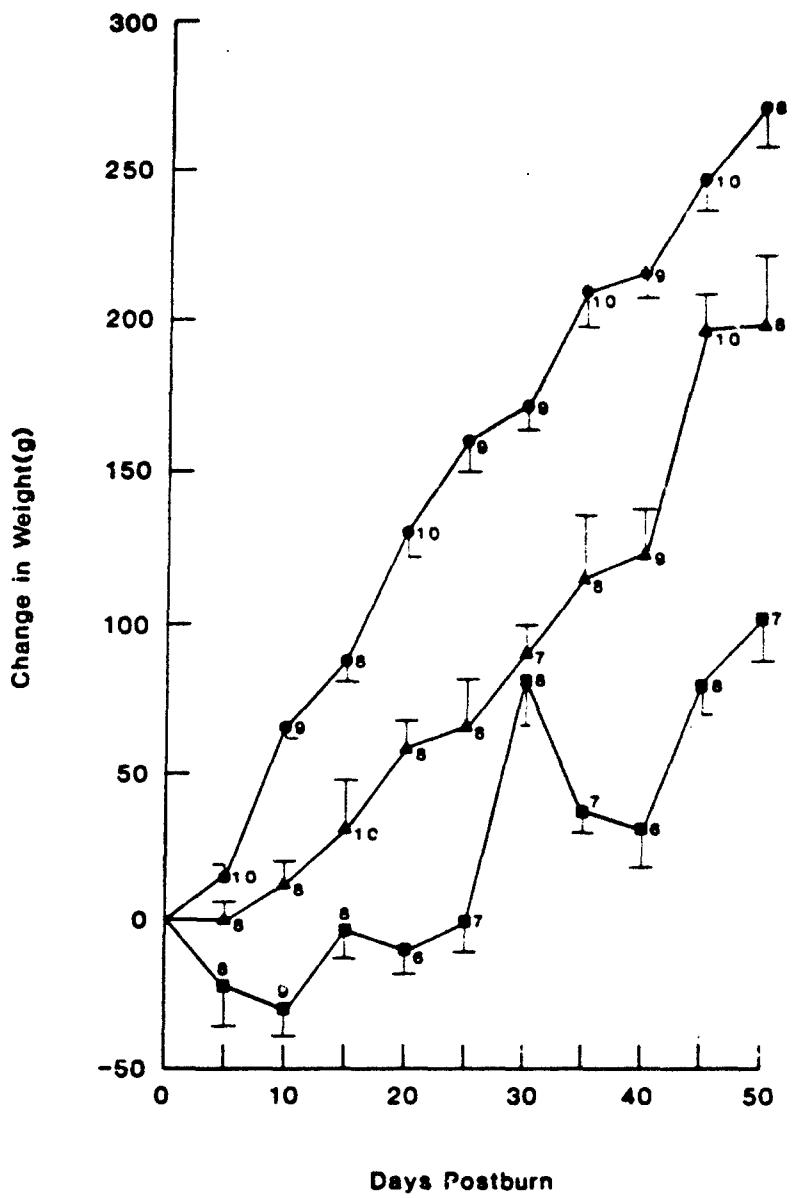


FIG. 1. Change in weight during 50 days following sham-burning (●) or burning of 15% (▲) or 30% (■) of the TBS. The points represent mean values, and each vertical bar represents the standard error of the mean (SEM). The numbers at each point specify animals from which weights were measured.

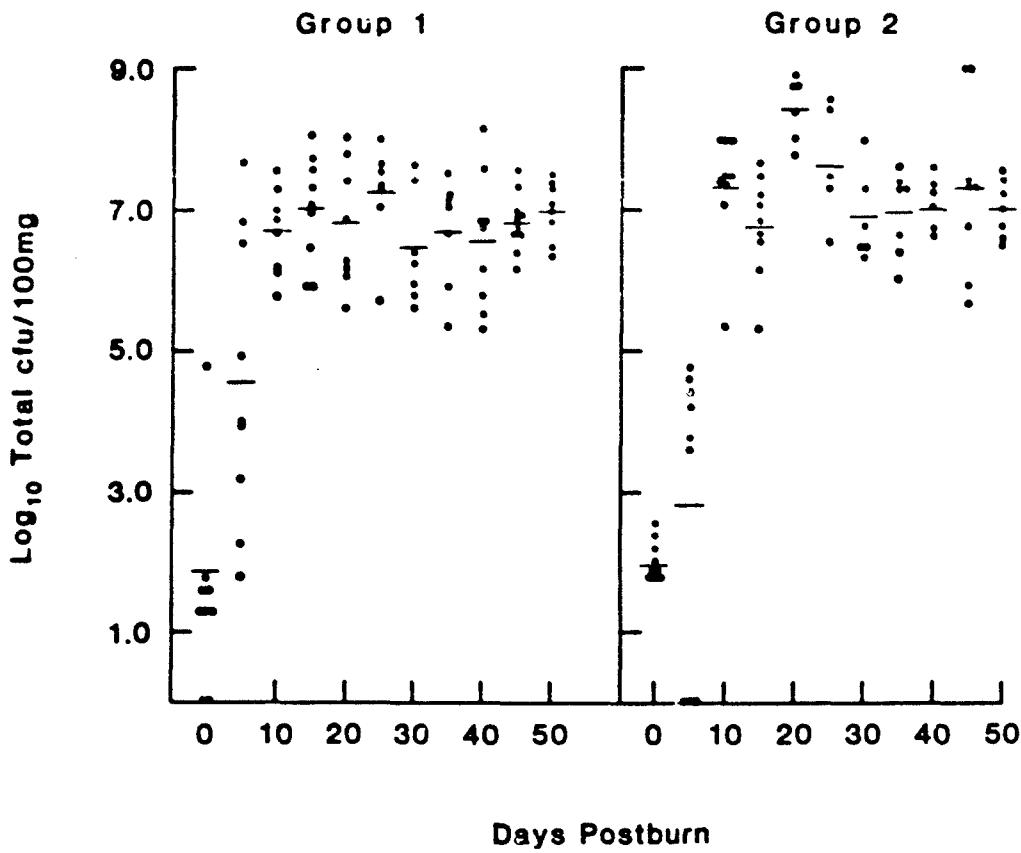


FIG. 2. Total cfu per 100 mg of burn wound tissue in animals with 15% (Group 1) and 30% (Group 2) TBS burns. The points represent the values obtained from all animals, and the horizontal lines represent mean values for each day postburn.

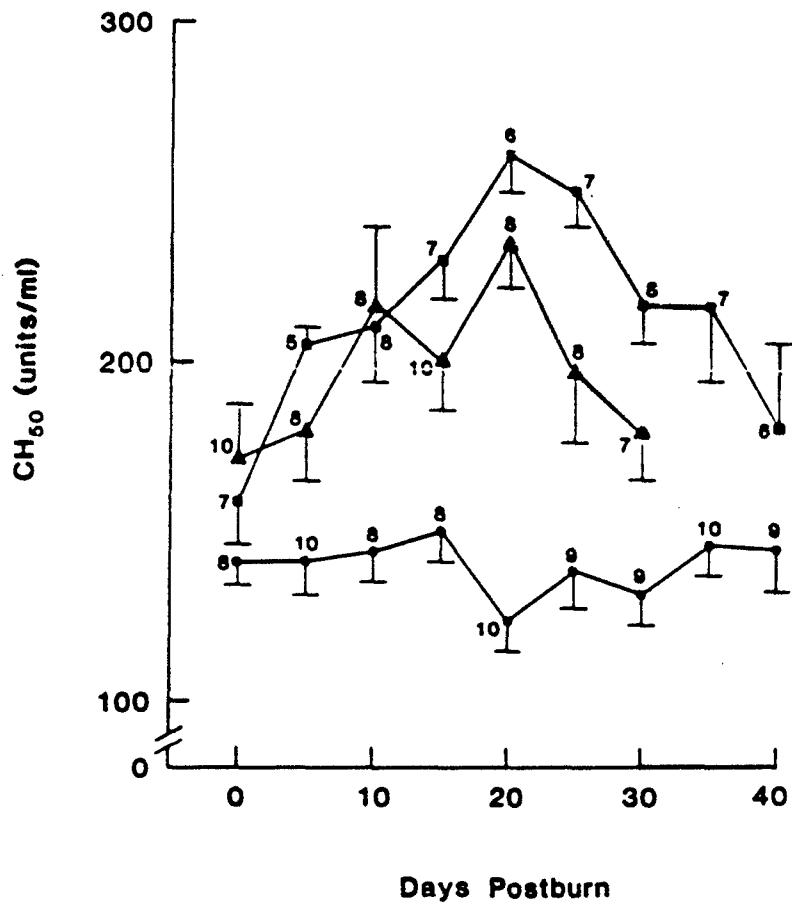


FIG. 3. Total hemolytic complement in the sera of guinea pigs during 40 days following sham-burning (●) or burning of 15% (▲) or 30% (■) of the TBS. The points represent mean values, and each vertical bar represents the SEM. The numbers at each point specify animals from which sera were analyzed.

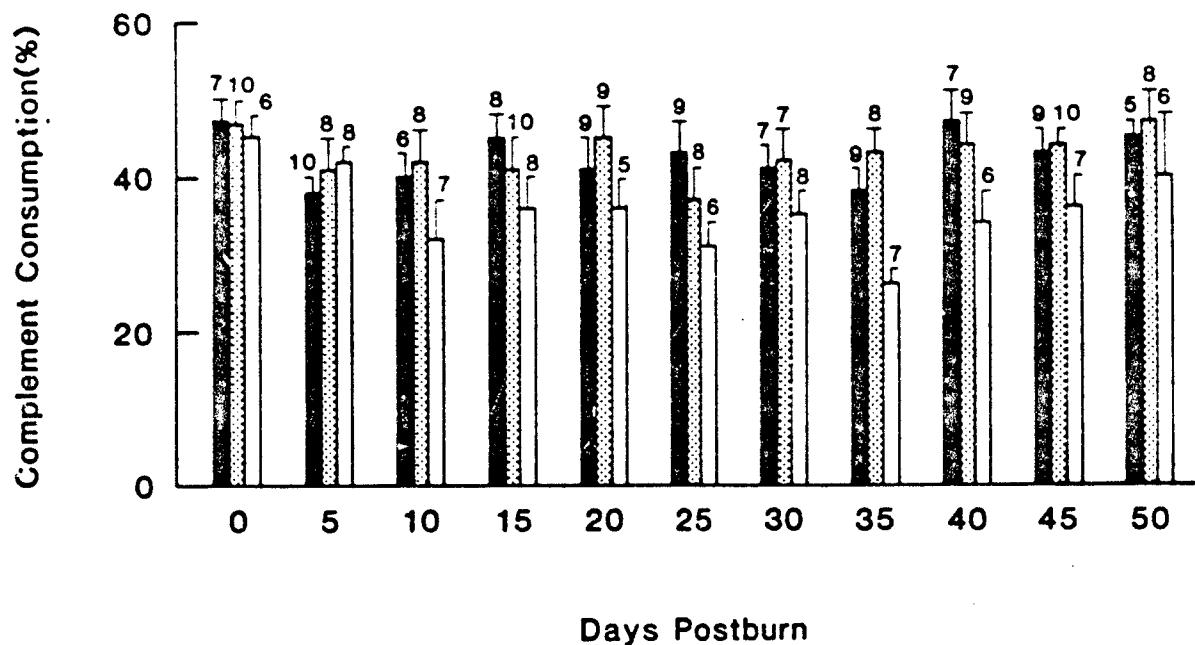


FIG. 4. Alternative pathway-mediated complement consumption by inulin in sera from animals with approximately 15% (▨) and 30% (□) TBS burns and sham-burned animals (■). The mean, SEM, and number of animals at each day postburn are shown.

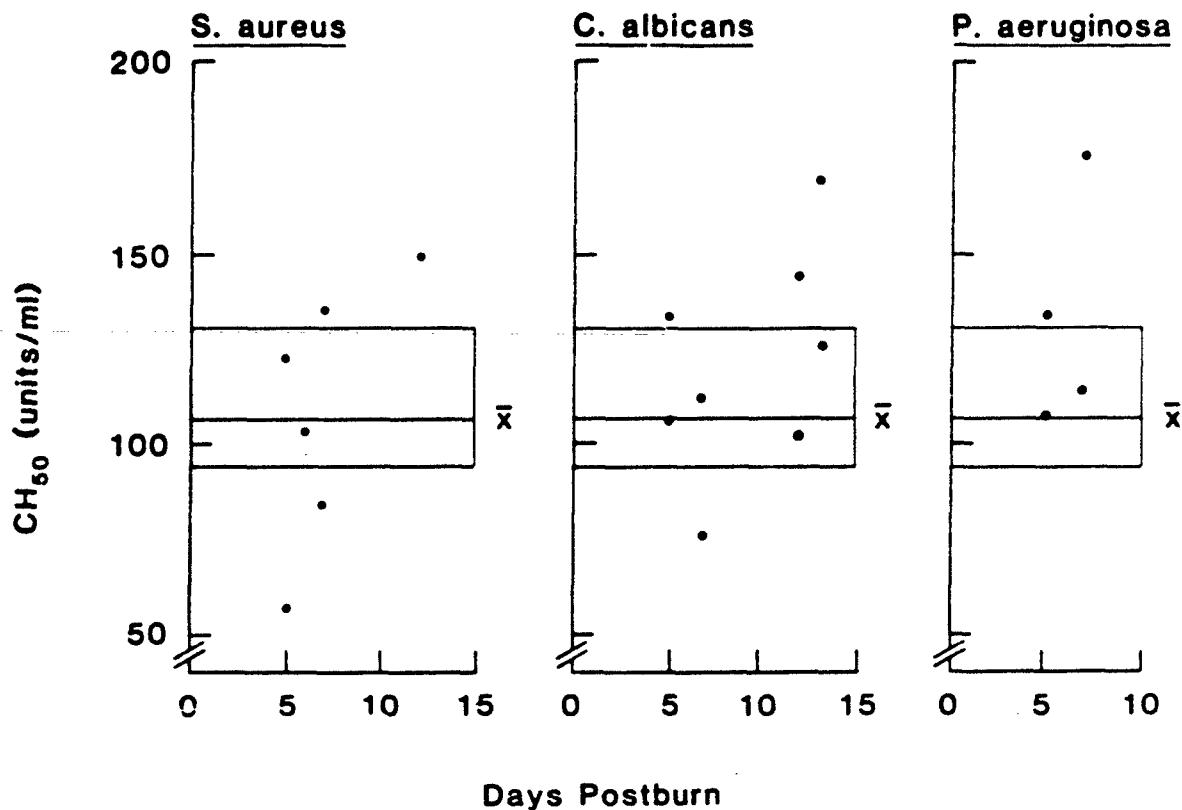


FIG. 5. Total hemolytic complement in the sera of burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

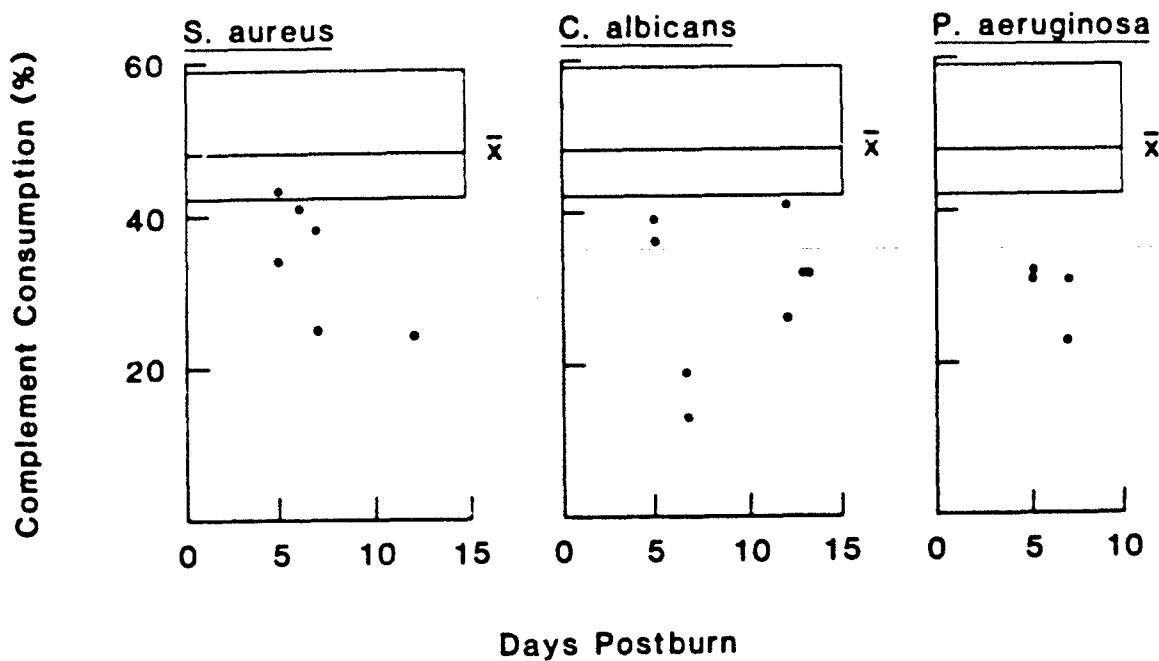


FIG. 6. Alternative pathway-mediated complement consumption by inulin in the sera of burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

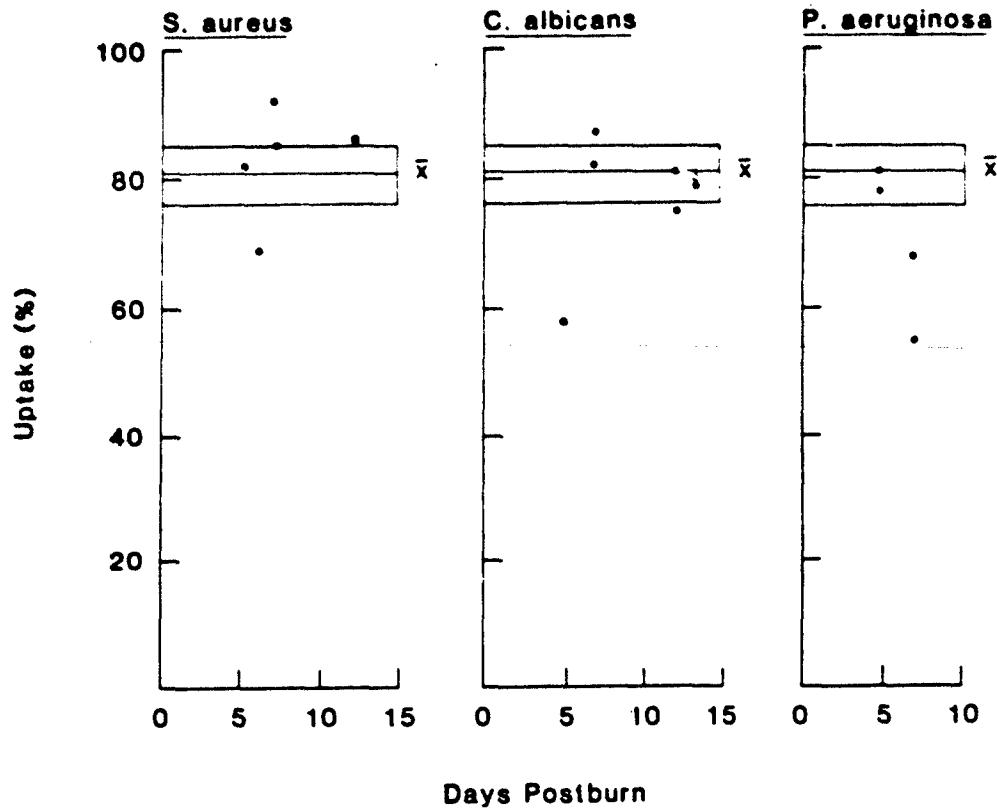


FIG. 7. Uptake of *E. coli* 075 by normal guinea pig peritoneal polymorphonuclear neutrophils in the presence of 10% of sera from burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

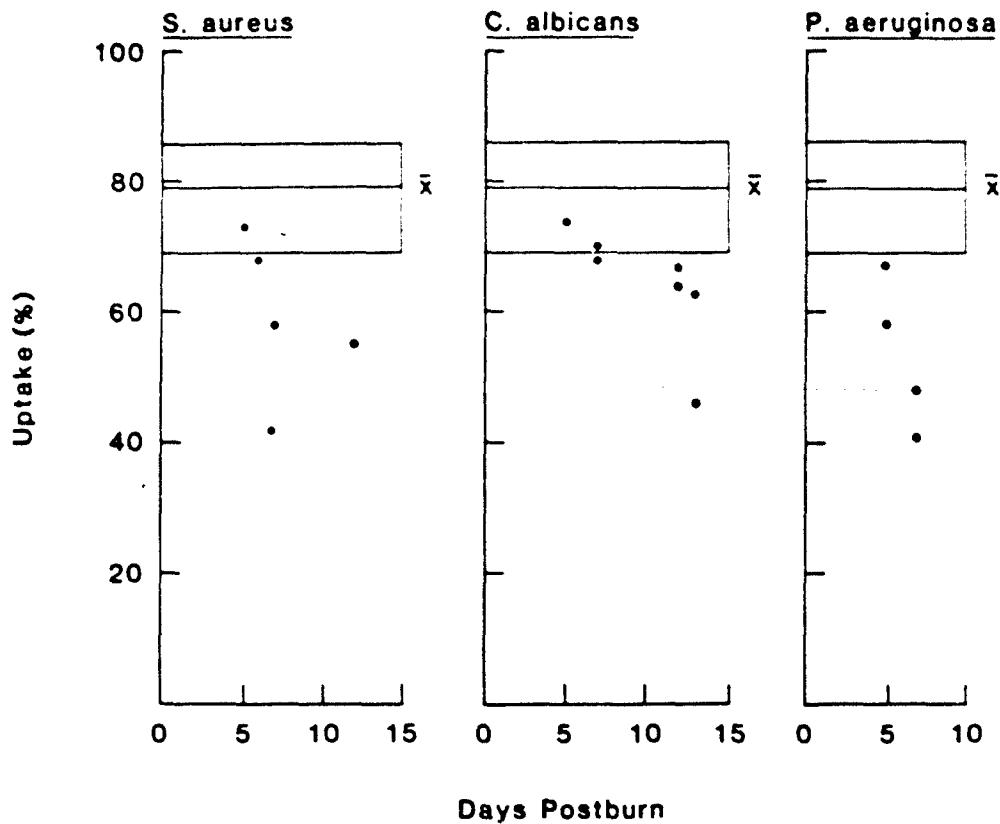


FIG. 8. Uptake of E. coli 075 by normal guinea pig peritoneal polymorphonuclear neutrophils in the presence of 98% of sera from burned animals experimentally infected with S. aureus, C. albicans, and P. aeruginosa. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

VII. DISTRIBUTION LIST

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